THE NATURE OF PROTON-TRANSLOCATING ATPases IN MAIZE ROOTS

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ABSTRACT: The mechanisms of the coupling between ATP hydrolysis and proton transport catalyzed by the ATPases of the tonoplast and plasma membrane of maize *(Zea mays L.)* roots were investigated. Proton transport by the tonoplast ATPase was found to be much more sensitive to nitrate than ATP hydrolysis, being inhibited by 80% with almost no effect on hydrolysis at 5 mM NO^3. Mercury was also found to be a potent inhibitor of this enzyme, inhibiting transport and hydrolysis by 50% at 60 and 100 μM, respectively. The same type of pattern was seen with other divalent cations. Millimolar concentrations of Cd^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} in the presence of Mg^{2+} inhibited proton transport significantly more than hydrolysis whereas Ba^{2+} and Ca^{2+} had little effect. Both free and ATP-complexed species of these inhibitory cations appeared to be effective. When the influence of temperature was investigated, both the tonoplast and plasma membrane enzymes showed a similar pattern. ATP hydrolysis by both enzymes generally obeyed the Arrhenius model, increasing in rate between 10°C and 45°C. However, the rate of proton transport deviated from this pattern above 20°C, remaining constant up to approximately 30°C and decreasing to undetectable levels by 40°C. In the presence of 50 μM vanadate, ATP hydrolysis by the plasma membrane ATPase was reduced by approximately 60% versus 30% for proton transport. In addition, vanadate significantly decreased the first order rate constant, ki, indicating a lowering of proton efflux. The carboxyl-modifying reagent N,N'-dicyclohexylcarbodiimide nearly abolished transport with only a 50% reduction in ATP hydrolysis. These data are interpreted in relation to whether the link between proton transport and ATP hydrolysis is direct or indirect for each enzyme.

INTRODUCTION

Membranes isolated from plant roots have been shown to contain at least two proton translocating ATPases (1,2). One of these enzymes is localized on the tonoplast membrane and is similar to other vacuolar type ATPases in that it is inhibited by N-ethylmaleiimide and nitrate, but insensitive to vanadate. This enzyme is probably involved in regulating the pH of the cytoplasm and mediating secondary transport processes across the tonoplast (3). The other enzyme is believed to be localized on the plasma membrane and shares the characteristics of the El-E2 type ATPase in forming an aspartyl phosphate intermediate, being sensitive to vanadate and utilizing Mg-ATP as substrate (4). The ATPase of the plasma membrane may also be involved in regulating cytoplasmic pH, in addition to mediating transport into and out of the cell (5).

According to the chemiosmotic theory (6), the electrochemical proton gradient is the main driving force for transporting various ions, amino acids, sugars and other molecules across membranes. In mitochondria and chloroplasts this gradient is formed by the energy derived from respiration and photosynthesis, respectively, whereas in other membranes including the tonoplast and plasma membrane of plants, hydrolysis of ATP provides the necessary energy via the proton ATPase.

There are two possible mechanisms by which ATPases may convert the energy of ATP hydrolysis into an electrochemical proton gradient: direct or indirect coupling. If the process leading to proton transport shares part of the molecular pathway responsible for ATP hydrolysis, then this is said to be direct coupling. An example of direct coupling is the redox-loop mechanism proposed for electron transfer-coupled proton movement in mitochondria (6). Alternatively, the primary energy-releasing process, *i.e.*, ATP hydrolysis, may be only indirectly linked to proton transport in that certain intermediate steps are required to couple ATP hydrolysis to the transport of protons.

In this paper, the nature of the coupling between ATP hydrolysis and proton translocation by the ATPases of the tonoplast and plasma membrane from corn roots was investigated utilizing kinetic analysis and other biochemical methods.

MATERIALS AND METHODS

Plant Materials: Three day old corn seedlings (*Zea mays L.* cv. FRB73 or cv. WF9 x Mo17) were grown on filter paper and harvested as described previously (7).

Preparation of Membrane Vesicles: Tonoplast-enriched vesicles were purified from roots of FRB73 by isopycnic density centrifugation (2). Briefly, 60 to 80 g fresh weight of roots were ground with mortar and pestle at 4°C in Grinding Buffer (GB) containing 0.3 M sucrose, 5 mM EDTA, 5 mM dithioerythritol, 5 mM 2-mercaptoethanol and 0.1 M NaHepes, pH 7.7. The homogenate was filtered through 4 layers of cheesecloth, and after differential centrifugation, a microsomal pellet was obtained which was resuspended in GB and overlayered onto a continuous sucrose gradient. The gradient was composed of 15 to 45% (w/w) sucrose made up in 5 mM Hepes-Mes, pH 7.7 plus 1 mM dithioerythritol. After centrifuging in a Beckman SW 28 rotor at 84,000g (average) at 4°C for 16 to 18 hours, the gradient was fractionated as described previously (8).

Membranes enriched in vanadate-sensitive ATPase activity were prepared from cv. WF9 x Mo17 by KI washing as outlined by Brauer et al. (7). In summary, a crude homogenate of roots from cv. WF9 x Mo17 was prepared by the same methods described above using a homogenization buffer (HB) containing 0.25 M sucrose, 10% (w/v) glycerol, 2 mM EGTA, 2 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 0.5% BSA, 5 mM dithiothreitol, and 25 mM Bis-Tris-Propane, pH 7.8. The microsomal pellet obtained after differential centrifugation, was resuspended in resuspension buffer (RB) composed of 0.25 M sucrose, 10% (w/v) glycerol, 2 mM dithiothreitol, and 2 mM Bis-Tris-Propane, pH 7.2. In some instances, a KI wash was performed by resuspending the original pellet in HB containing 0.25 M KI, incubating on ice for 10 minutes, then pelleted and resuspended in RB. The vanadate-sensitive ATPase was reconstituted by a detergent dilution method as previously described (7).

Measurement of Proton Transport: Proton transport was followed by changes in the absorbance of acridine orange (AO) at 492 nm as described by de Michaelis *et al.* (9). Typically, 50 to 200 µL of membrane vesicles were diluted with 2 mL of 25 mM D-glucose, 2.5 mM $MgSO_4$, 1 mM EGTA, 7.5 pM AO, 50 mM KCl or KNO_3, and 17.5 mM Mes-BTP (pH 6.45). After a period of equilibration, transport was initiated by the addition of 20 µL of 0.2 M Na_2ATP (final concentration = 2.0 mM).

Kinetic Analysis of Proton Transport: The kinetic properties of proton transport were analyzed using a model previously developed in the study of proton transport by membranes of purple bacteria (10) and modified for plant membranes

(2). The rate of net proton transport to the interior of membrane vesicles at a given time may be expressed mathematically as

$$d\partial/dt = mR - (k_L + k_{bp}) = mR - k_i \cdot \partial \quad [1]$$

in which ∂ is the net amount of protons transported across the membrane at time **t**, after the addition of ATP, and **R** is the rate of ATP hydrolysis by the pump. The coefficient m is a measure of the coupling between ATP hydrolysis and proton transport. The rate constants k_L and k_{bp} represent membrane leakage during transport and the back pressure effect, respectively. The sum of these two constants, k_i represents the processes which inhibit the buildup of the proton gradient. The quantity ∂ can be directly related to changes in absorbance of the dye AO (11). At steady state, the net rate of proton transport, $d\partial/dt$, approaches zero and therefore,

$$mRs = k_i \cdot \partial s \quad [2]$$

where the subscript s denotes the steady state level. If ATP hydrolysis remains unchanged, the initial rate of ATP hydrolysis can be substituted for R_S. Application of the steady state approximation yields

$$mR_S = mR_o = mR = k_i \cdot \partial s \quad [3]$$

where the subscript o denotes the initial rate, and Equation [1] reduces to

$$d\partial/dt = k_i(\partial - \partial_s) \quad [4]$$

Upon integration of Equation [4], the following is obtained

$$\ln (1 - \partial/\partial_s) = -k_i t \quad [5]$$

Therefore, Equation [5] provides a convenient means of estimating ki, the rate constant that represents the processes which inhibit the formation of the proton gradient. By replacing ∂ with the change in absorbance of AO, one can plot the natural log of 1 minus (the decrease in absorbance at time t)/(the total decrease in absorbance at steady state) as a function of time after the addition of ATP. The slope of the line will be ki and using Equation [3] one can calculate the initial rate of proton transport by:

$$R_H = k_i \partial_s \quad [6]$$

where R_H, the initial rate of proton transport, is equal to **mR**.

ATP Hydrolysis Assays: ATP hydrolysis was assayed using 5 to 10 μL of vesicles diluted to 100 μL with reaction media used for assaying proton transport without acridine orange. In some cases, aliquots from the actual proton transport assays were used to determine phosphate released. The amount of inorganic phosphate released was determined by the formation of the malachite green-phosphomolybdate complex after terminating the reaction with the addition of ice-cold 5% TCA and incubating on ice (2). Alternatively, the rate of ATP hydrolysis was determined by monitoring the changes in absorbance at 340 nm following the oxidation of NADH in the presence of pyruvate kinase, lactate dehydrogenase and phosphoenolpyruvate as described previously (2). ATP hydrolysis by microsomes was determined in the absence and presence of 0.2 mM vanadate and the difference was attributed to the plasma membrane-type ATPase.

Protein Determination: Protein was assayed using either the Bradford (12) or modified Lowry method (13) using BSA as a standard.

RESULTS AND DISCUSSION

Effects of Inhibitors on the Tonoplast ATPase: The effects of nitrate on proton transport and ATP hydrolysis by the tonoplast enzyme are shown in Figure 1. The initial rates of proton transport and ATP hydrolysis were determined at a constant K^+ concentration and ionic strength, but varying the ratio of chloride to nitrate ions in the assay medium. Proton transport showed a much greater sensitivity to nitrate than ATP hydrolysis, being inhibited by 80% at a nitrate mole fraction of only about 0.07 with almost no effect on hydrolysis.

A similar differential effect on the hydrolytic and transport activities of the tonoplast ATPase is seen with the Hg^{2+} ion (Fig. 2). Hg^{2+} is quite effective at micromolar concentrations, with hydrolysis and proton transport inhibited by 50% at approximately 100 and 50 pM, respectively. Complete inhibition of both processes occurred at 400 pM. As with nitrate, Hg^{2+} preferentially inhibits proton transport over ATP hydrolysis, although Hg^{2+} is a much more potent inhibitor. Also, *p*-hydroxymercuribenzoate had inhibitory properties similar to Hg^{2+} (data not shown).

Effects of Divalent Cations: The influence of other divalent cations on the coupled activities of the tonoplast ATPase was investigated. Of the cations tested, which included Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, only Mn^{2+} was able to replace Mg^{2+} in supporting ATP hydrolysis and proton transport (14).

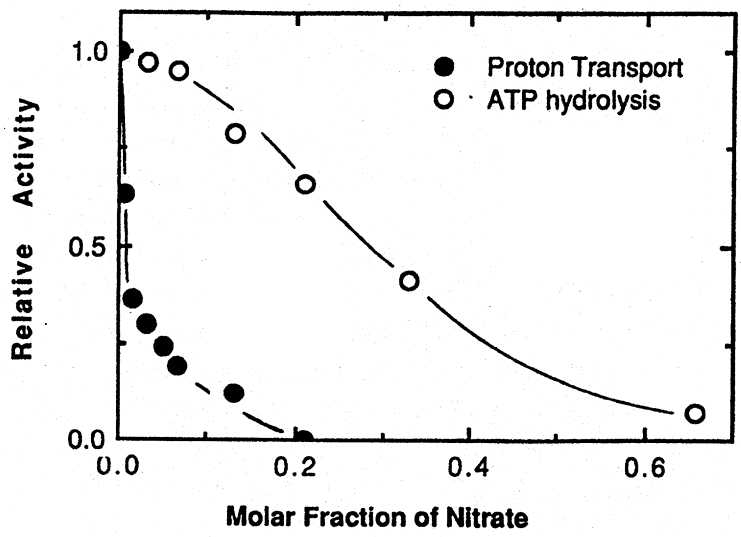

FIGURE 1. Differential Inhibition of ATP Hydrolysis and Proton Transport Activities of the Tonoplast-type ATPase by Nitrate. Tonoplast membrane vesicles were incubated in assay solution in which a portion of the KCl has been replaced with KNO_3, and assayed for proton transport (●) and ATP hydrolysis (o). Data are plotted relative to samples assayed in the absence of nitrate, which averaged 1.8 A/ min/ mg protein and 1080 nmol Pi/ min/ mg protein, respectively.

In the presence of an optimal Mg^{2+} concentration, Ca^{2+} and Ba^{2+} had little effect on both types of activity, whereas Cd^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} inhibited both hydrolysis and proton transport (data not shown). Figure 3 shows representative data of the effects of one of these inhibitory cations, Cd^{2+}. Again, a differential effect was seen with proton transport being more sensitive.

Cadmium (Cd^{2+}) concentrations as low as 0.05 mM inhibited proton transport by more than 50%, whereas ATP hydrolysis was only slightly affected. The effects of the inhibitory cations were evaluated in terms of the relative contributions of the free versus ATP-complexed species utilizing equilibrium analysis. A complex pattern involving inhibition by both species was found which is discussed in detail in a previous paper (14).

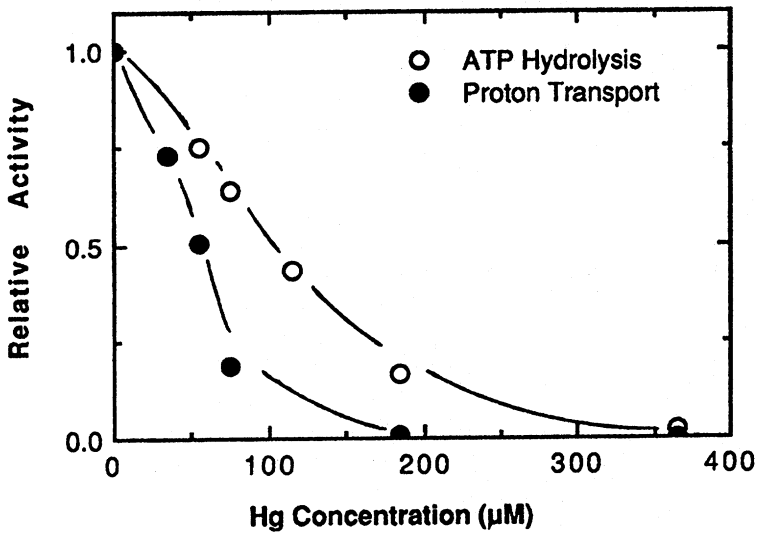

FIGURE 2. Differential Inhibition of Proton Transport and ATP Hydrolysis Activities of the Corn Root Tonoplast ATPase by Hg^{2+}. Tonoplast vesicles were incubated in the presence of Hg^{2+} in assay solution for 10 min before assaying ATP hydrolysis (o) and proton transport (●). The data are plotted relative to vesicles incubated in the absence of Hg^{2+} which averaged 980 nmol Pi/ min/ mg protein and 1.8 A/ min/ mg protein.

Effects of Temperature on the Tonoplast ATPase: The temperature dependence of proton transport and ATP hydrolysis was evaluated between 5 and 45°C (Fig. 4). With increasing temperature the initial rate of ATP hydrolysis increased in an exponential manner. On the other hand, the initial rate of net proton transport exhibited a bell-shaped pattern, with the maximum located between 25 and 30°C. The ratio of proton transport to ATP hydrolysis remained nearly constant at temperatures below 20°C. However, above this temperature, the ratio decreased. To investigate more thoroughly the differential effect of temperature on proton transport and ATP hydrolysis, the kinetics of these two processes were analyzed by calculating the respective Michaelis-Menten constants (Km) at the

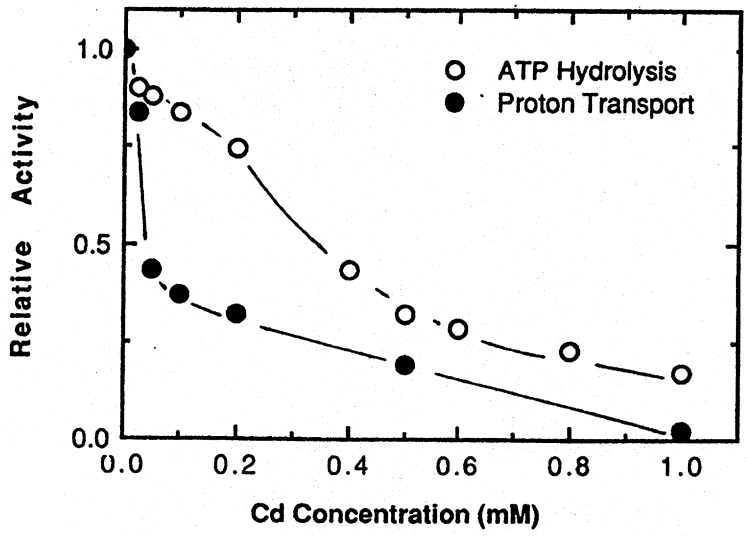

FIGURE 3. Effects of Cd Concentration on the Activities of the Tonoplast ATPase. Tonoplast vesicles were incubated in assay buffer with varying concentrations of $CdCl_2$ for 6 min and then assayed for proton transport (•) and ATP hydrolysis (o). Data are plotted relative to controls incubated in the absence of Cd which averaged 2.1 A/ min/ mg protein and 301 nmol Pi/ min/ mg protein, respectively.

different temperatures. The Km for ATP hydrolysis was relatively constant over the temperature range. In contrast, the Km for proton transport increased dramatically with increasing temperature (Table 1). These results indicate that certain molecular processes which contribute to the kinetics of transmembrane proton transport are not directly involved in the pathway leading to the hydrolysis of ATP.

Effects of Inhibitors on the Plasma Membrane ATPase: In reconstituted KI-washed microsomes where almost all of the ATPase activity can be attributed to the vanadate-sensitive ATPase (7), ATP hydrolysis was more sensitive to inhibition by vanadate than proton transport (Table 2). In the presence of 0.05 mM vanadate, the rate of ATP hydrolysis was lowered by almost 60% as compared to only a 30% reduction in $\mathbf{R_H}$, the initial rate of proton transport. The

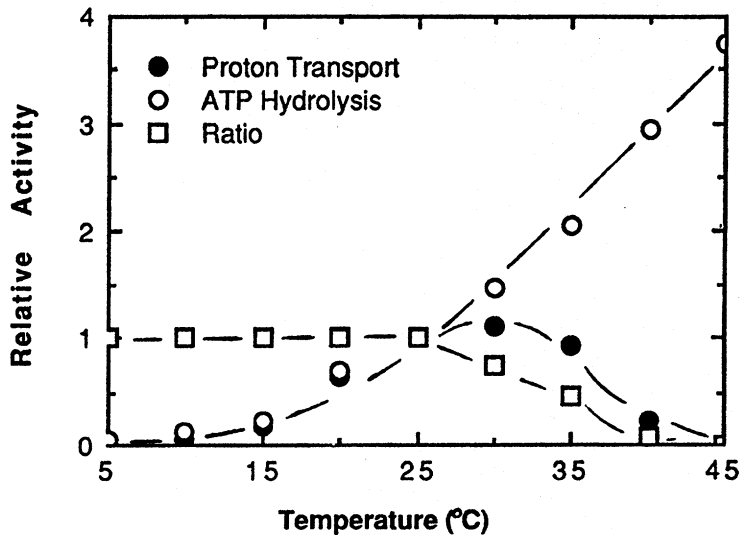

FIGURE 4. Temperature Dependence of Tonoplast ATPase Activities. Tonoplast vesicles were assayed for proton transport (●) and ATP hydrolysis (o) at varying temperatures between 5 and 45° C. Data are plotted realtive to the activities at 25 C, which averaged 1.86 A/ min/ mg protein and 1050 nmol Pi/ min/ mg protein, respectively. From these data, the ratio of proton transport to ATP hydrolysis was calculated (□).

reason why proton transport was inhibited less than ATP hydrolysis is because the two components comprising R_H, *i.e.* ∂_s and k_l, were inhibited less than ATP hydrolysis.

Sussman and Slayman (15) have demonstrated that the *Neurospora* plasma membrane ATPase is quite sensitive to N,N'-dicyclohexylcarbodiimide (DCCD). They found that inactivation of ATP hydrolysis is a function of time as well as concentration. The plasma membrane ATPase of corn roots was also inhibited by DCCD (Fig. 5). When reconstituted plasma membrane ATPase was incubated with various concentrations of DCCD, proton transport showed a more pronounced sensitivity to DCCD inhibition than ATP hydrolysis. At 30 pM

TABLE 1. Km for ATP for Proton Transport and ATP Hydrolysis Catalyzed by Tonoplast ATPase at Different Temperatures.

Temperature (°C)	ATP Hydrolysis	Proton Transport
	mM	mM
10		0.26 + 10 %[a]
15	0.14 + 10 %	
20		0.47 + 5 %
25	0.18 + 10 %	
30		0.77 + 5 %
35	0.16 + 10 %	
40		1.32 + 5 %

[a] Data are the average of two trials, plus or minus the coefficient of variation.

DCCD, most of the transport activity was abolished, while the rate of ATP hydrolysis was 50% of the control.

Temperature Dependence of the Plasma Membrane ATPase: When ATP hydrolysis and proton transport by the vanadate-sensitive ATPase were assayed over a temperature range of 7°C to 45°C, a differential effect similar to that seen with the tonoplast ATPase was observed (Fig. 6). The rate of ATP hydrolysis by the plasma membrane ATPase increased throughout the temperature range. When these data were plotted according to the Arrhenius equation, a straight line was obtained (data not shown). The activation energy estimated from the slope of this line was calculated as 14.5 kcal/mole, a value similar to that obtained with the tonoplast ATPase. Data for proton transport, however, did not follow the same pattern. Below 21°C, R_H increased with increasing temperature. After remaining relatively unchanged between 21°C and 29°C, R_H decreased with increasing temperature until at 39°C there was no detectable transport. This

TABLE 2. Effects of Vanadate on the Coupled Activities of the Plasma Membrane ATPase.

[Vanadate]	R	Proton Transport R_H	δ_S	k_i
mM	Relative Activity [a]			
0.05	0.42	0.72	0.79	0.91
0.10	0.21	0.43	0.51	0.84
0.20	0.05	0.25	0.33	0.74
0.40	0.00	0.00	0.00	0.00

[a] Data for ATP hydrolysis (R), inital rate of proton transport (R_H), extent of proton transprt at steady-state (δ_S) and rate constant for inhibitory process (k_i) are plotted to the values obtained in the absence of vanadate, which averaged 91 nmol/ min/ mg protein, 0.12 A/ min/ mg protein, 0.33 A/mg protein and 0.36 min^{-1}, respectively.

divergence in behavior between hydrolysis and transport leads to a decrease in the ratio between the rates of these two processes, suggesting an effect on the coupling mechanism. As with the tonoplast ATPase, the decrease in R_H was accompanied by an increase in k_i (data not shown), an indicator of a change in membrane properties. Thus, the decrease in the initial rate of proton transport could be due to an interaction of the lipid bilayer with a part of the domain involved in proton transport which is distinct from that involved in ATP hydrolysis.

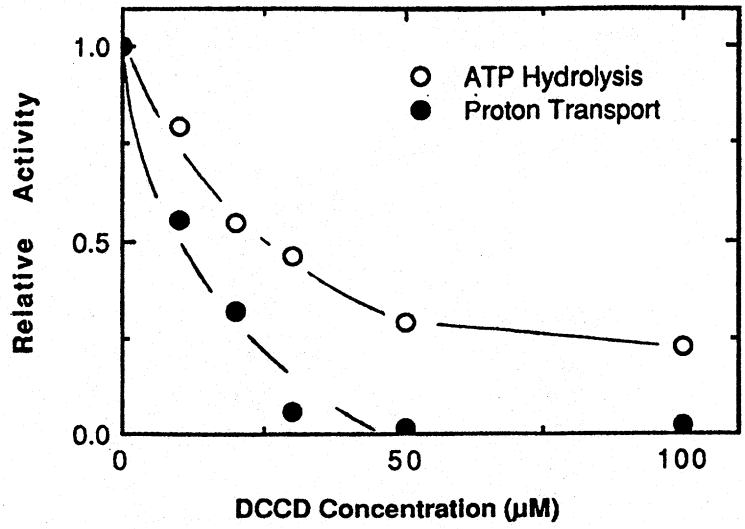

FIGURE 5. Effect of Different Concentrations of DCCD on the Activities of Reconstituted Plasma Membrane ATPase. Reconstituted plasma membrane ATPase was incubated with different concentrations of DCCD for ten minutes and then assayed for proton transport (●) and ATP hydrolysis (o). Data are plotted relative to untreated membranes, which averaged 0.15 A/ min / mg protein and 160 nmol Pi/ min/ mg protein, respectively.

CONCLUSIONS

Both the tonoplast ATPase and the plasma membrane ATPase function in transporting protons out of the cytoplasm of plant root cells. The transmembrane electrochemical potentials thus generated are believed to be the primary force for secondary ion transport processes (3,5). Despite their similarity in function, the two ATPases nevertheless possess fundamental differences in structure and reaction mechanism. The plasma membrane ATPase is composed of only one type of polypeptide (Mr ≈ 100,000) and, as mentioned before, forms a phosphorylated intermediate during catalysis (4,16). On the other hand, the tonoplast ATPase is made up of at least three different subunits and does not form a phosphorylated intermediate during the reaction cycle (3).

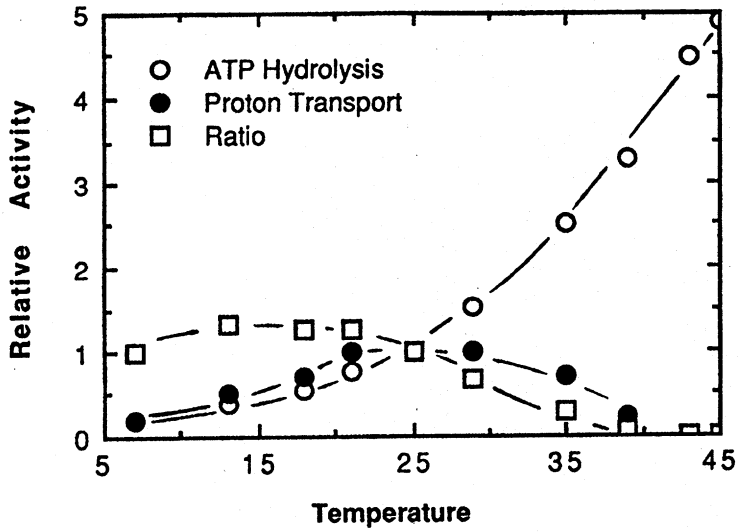

FIGURE 6. Effect of Temperature on the Activities of Plasma Membrane ATPase. KI-washed microsomes were assayed for proton transport (●) and vanadate-sensitive ATP hydrolysis (o) at varying temperatures between 7 and 45 ° C. Data are plotted relative the the activities at 25 C, which averaged 0.12 A/ min/ mg protein and 160 nmol Pi/ min / mg protein, respecitvely. From these data, ratios of proton transprot to ATP hydrolysis (□) were calculated.

Interestingly enough, both enzymes have a very similar dependence on temperature. As with the tonoplast ATPase, the Km for ATP hydrolysis by the plasma membrane ATPase is relatively insensitive to temperature in the range of 10 to 40°C (data not shown) with the activation energy for the process calculated to be between 14 and 15 kcal/mole (Fig. 6).

Among the membrane ATPases of plant cells, the reaction mechanism of the tonoplast ATPase is probably the least understood. Some of its properties, such as the lack of a phosphorylated intermediate, are similar to the mitochondrial enzyme. Although there is little information to firmly establish the molecular coupling mechanism between ATP hydrolysis and proton translocation in the tonoplast

system, the differential effects of inhibitors and temperature as shown in this paper suggest that the coupling is indirect in nature. Accordingly, at least some intermediate chemical or conformational steps are required for the coupling of these two events. Using the indirect coupling model proposed for the mitochondrial ATPase (17) and bacteriorhodopsin (18) as a conceptual guide, the effects of chemical events occurring at the catalytic site in a protein domain (domain I) of the tonoplast ATPase may be propagated through a specific link to trigger proton translocation by a "protogenic domain" (domain II) which may or may not be an integral part of the ATPase. The observed preferential inhibition of proton transport suggests that inhibitors, such as nitrate, Hg^{2+} or divalent cations, may interact with the protogenic domain to cause it to be less responsive to ATP hydrolysis at the catalytic site, as reflected by a decrease in the coupling factor, m. At higher inhibitor concentrations, the link between the two domains is completely blocked. Consequently, the turnover of the enzyme cannot be completed and the result is an inhibition of ATP hydrolysis.

As in the case of the tonoplast ATPase, it is possible that the enzyme of the plasma membrane may also possess an indirect coupling mechanism. The differential effects of DCCD and vanadate on the hydrolytic and transport activities of this ATPase supports this hypothesis. Unlike the tonoplast ATPase where proton transport displayed a greater sensitivity to all the inhibitors tested, the relative sensitivities of the coupled activities of the plasma membrane ATPase are dependent on the inhibitor used. Proton transport is more sensitive to DCCD whereas ATP hydrolysis is more strongly inhibited by vanadate. This is consistent with what is known about the modes of action of these compounds (15). Effectiveness of vanadate as an inhibitor of El-E2 ATPases is believed to be due to its structural similarity to phosphate, acting as a transition state analogue during the part of the reaction cycle involving dephosphorylation (19,20). Strictly, a direct coupling mechanism would suggest an obligatory interaction between the two processes such that a constant stoichiometry of ATP hydrolyzed to protons transported would exist. In other words, the turnover of one process is totally dependent on the turnover of the other. Because this does not appear to be the case with the plasma membrane ATPase, an indirectly-coupled mechanism similar to that proposed for the tonoplast ATPase is implicated. Of course, more experimental evidence is required to clarify this point.

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